12-Hydroxy-5,8,10,14-Eicosatetraenoic Acid (12-HETE) Does Not Stimulate Proliferation of Human Neonatal Keratinocytes

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We have developed an assay to study the effect of drugs on the proliferation of neonatal human skin-derived keratinocytes in vitro. Expanding populations of neonatal keratinocytes were cultured in low concentrations (0.5%) of fetal calf serum for up to 12 d. Growth of the cultures was determined by measurement of DNA using a sensitive fluorimetric assay. Addition of 10^{-9}–10^{-6} M 12(RS)-hydroxy-5,8,10,14-eicosatetraenoic acid (12(RS)-HETE) neither stimulated keratinocyte proliferation nor enhanced the incorporation of [^{3}H]thymidine. The ability of neonatal keratinocytes in low serum medium to respond to exogenous factors was demonstrated by increased growth in response to a mixture of cholera toxin, hydrocortisone, and epidermal growth factor. Confluent keratinocyte cultures in 10% human AB serum exposed to 12(S)-HETE for 72 h also showed no changes in DNA, [^{3}H]thymidine incorporation, or labeling index. Metabolism of 12(S)-[^{3}H]HETE was greater in cultures containing low concentrations of serum but there was no evidence for the formation of 12,20-dihydroxyeicosatetraenoic acid. *J Invest Dermatol 92:683–688, 1989*

Increased levels of arachidonic acid and its metabolites, particularly the lipoxygenase products, are a feature of the lesional skin of psoriasis [1–4]. The 12-lipoxygenase pathway appears to predominate in adult human epidermis in vitro [5], although it is also reported that 15-lipoxygenase activity is higher in neonatal skin keratinocytes [6]. The analysis of scale and exudates from psoriatic and normal skin has shown that 12-hydroxy-5,8,10,14-eicosatetraenoic acid (HETE) is the major arachidonate lipoxygenase product in vivo [7,8]. In psoriasis there is approximately a threefold increase in the amount of 12-HETE recovered in exudates of the lesional skin compared with exudates from either uninvolved skin or skin of healthy subjects [2].

The proinflammatory properties of 12-HETE in human skin are well documented [9–11]. Epicutaneous application to human skin causes erythema, edema, infiltration of the dermis with neutrophils and mononuclear cells, and migration of neutrophils into the epidermis with the formation of epidermal microabscesses resembling the histopathologic changes seen in the early stages of developing psoriatic lesions [12]. The relationship between increased arachidonic acid lipoxygenation and other features of psoriasis, particularly epidermal hyperplasia, is not clear. 12(S)-HETE [13] and the 5-lipoxygenase products, LTB_4, LTC_4, and LTD_4 [14], are reported to stimulate [^{3}H]thymidine incorporation into confluent adult human keratinocyte cultures. An increase in DNA was not demonstrated in these studies and it is, therefore, unsafe to conclude that an increased rate of keratinocyte proliferation occurred on exposure of the cells to the lipoxygenase products.

Growth assays have been subject to controversy [15]. The accurate determination of growth rates in expanding cell populations is dependent on demonstrating an increase in cell numbers or a rise in DNA. For a steady state (confluent) population it is necessary to show an increased turnover of cells through the system. Reliance on [^{3}H]thymidine incorporation in the absence of prior calibration against cell numbers or DNA content may lead to spurious results [16–19]. In the present study we have investigated the effects of 12-HETE on the proliferation of human neonatal keratinocytes using a sensitive DNA assay as an end point. Expanding and confluent cultures were studied. In addition, we substantiated our results with measurements of [^{3}H]thymidine incorporation, cell counts, and labeling indices.

Conditions for keratinocyte culture based on the methods of Rheinwald and Green [20] were developed to encourage rapid proliferation and high yields of cells and as such are unsuitable for growth assays. We established a growth factor assay using low concentrations of serum for culture of neonatal skin-derived keratinocytes that limited cell proliferation but in which the cells retained their ability to proliferate rapidly in response to a mixture of specific exogenous factors.

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Abbreviations:
DMEM: Dulbecco's modified Eagle's medium
EDTA: ethylenediaminetetraacetic acid
EGF: epidermal growth factor
FCS: fetal calf serum
HEPES: *N*-2-hydroxyethyl-piperazine-*N*′-2-ethanesulfonic acid
HETE: hydroxyeicosatetraenoic acid
HPLC: high performance liquid chromatography
LT: leukotriene
M199: medium 199
PBS: phosphate-buffered saline

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MATERIALS AND METHODS

Preparation of Keratinocyte Suspensions Neonatal foreskin excisions were collected in medium 199 (M199) containing 25 mM *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid (HEPES), kanamycin, penicillin (each 100 μg/ml), and amphotericin B (1.25 μg/ml) and used within 24 h. After three washes in M199, the skin was incubated at 37°C in two changes of the same medium for 15 min each and washed again. Excess dermal tissue was removed and the skin cut into 1-mm strips, which were incubated in 0.5 mM EDTA in phosphate-buffered saline (PBS) [Glasgow formula (g/L), NaCl 8.0, KCl 0.2, $Na_2HPO_4 \cdot 12H_2O$ 2.9, KH_2PO_4 0.2, EDTA · Na_2 0.2, and 5 mg/L phenol red, pH 7.4] containing 2.5 mg/ml trypsin and antibiotics for 15 h at 4°C. The tissue was transferred to Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), kanamycin, amphotericin-B, and 1 mM pyruvate, and the epidermis and dermis separated with forceps. Both components were gently agitated to release the epidermal cells, which were filtered through a 100-mesh stainless steel gauze and collected by centrifugation at 150 *g* for 5 min at room temperature. The cells were washed twice in the same medium, counted in a hemocytometer, plated in flasks at 50,000 basal cells/cm², and incubated in humidified air containing 7.5% CO_2 at 37°C. The plating efficiency was approximately 1% of the basal cells. Nonadherent cells were removed at 24 h and subsequent medium changes were at 2–3-d intervals until confluence was reached at 7–10 d. Cells were dissociated by washing twice in 0.5 mM ethylenediaminetetraacetic acid (EDTA) in PBS and incubating in 0.5 mg/ml trypsin in buffered 0.5 mM EDTA for approximately 5 min at 37°C, and then washed twice in DMEM containing 10% FCS. The keratinocyte suspensions were used directly in experiments or cryopreserved in M199 containing 10% dimethyl sulphoxide under liquid nitrogen. Frozen cells were rapidly thawed at 37°C and washed with DMEM containing 10% FCS, antibiotics, and pyruvate before use.

Keratinocyte Growth Factor Assays Keratinocytes were plated at 18,000 basal cells/cm² in 24 well plates in 1 ml DMEM containing 10% FCS, antibiotics, and pyruvate. Nonadherent cells were removed at 24 h and the monolayers washed with serum free DMEM before continuing culture in DMEM containing 0.5% FCS. The 12-HETE was added from a 1000× concentrate stock solution in ethanol. Control cultures received ethanol alone to the same final concentration of 0.1%, or were supplemented with ethanol and a mixture of cholera toxin (10^{-10} M), hydrocortisone (0.4 μg/ml), and human epidermal growth factor (EGF) (10 ng/ml) as control. In addition, further control keratinocyte monolayers were cultured in DMEM with 10% FCS to demonstrate maximal growth. The culture media were changed on alternate days for periods up to 2 wk. Growth was assessed at intervals by assay of DNA or incorporation of [³H]thymidine.

Immunocytochemical Identification of Keratinocytes Second passage keratinocytes grown on glass or plastic coverslips for 3 d were fixed in acetone (glass) or ethanol (plastic) at −20°C for 10 min. The fixed cells were incubated overnight at 4°C with mouse monoclonal antibodies to cytokeratins AE1 (50 and 56 kD keratins), AE2 (56 and 65–67 kD keratins), and AE3 (58 and 65–67 kD keratins) [21], to desmin and vimentin, followed by incubation for 1 h in rabbit anti-mouse-HRP using the method described by Kirkland [22].

Mycoplasma Testing Cultures were routinely tested for mycoplasma contamination by the fluorescent dye binding method of Chen [23]. Cells grown on coverslips were fixed in Carnoys fixative, stained with 0.4 μg/ml bisbenzamide (Hoechst 33258) for 1 min, and examined using a Leitz Dialux 22 epifluorescence microscope. No positives were found.

DNA Assay Cell monolayers for DNA assay were washed twice with 1 ml PBS per well, drained, and stored frozen at −20°C until analyzed. DNA was measured using a modification of the fluorimetric method of Karsten and Wollenberger [24]. The frozen monolayers were thawed, incubated with 1.8 ml/well PBS containing 20 μg/ml bacterial pronase for 20 min at 37°C, and homogenized in situ on ice using a Polytron (Kinematica, Northern Media Supplies, U.K.) fitted with a 7-mm probe at speed setting 4.5 for 20 sec. Two 0.8-ml samples were transferred to plastic tubes to which were added 100 μl each of 115 μg/ml pronase and 125 μg/ml RNase in PBS. The tubes were incubated for 60 min at 37°C and 0.5 ml of 15 μg/ml ethidium bromide in PBS then added. Fluorescence was measured on a Perkin-Elmer LS-2 filter fluorimeter with excitation and emission wavelengths of 540 and 580 nm, respectively. The assay was standardized against calf thymus DNA (0–10 μg/ml) treated similarly.

[³H]Thymidine Incorporation Into DNA Cell monolayers were incubated with 1 μCi/ml methyl-[³H]thymidine for 18 h. Incorporation was measured by the method of Dicker and Rozengurt [25]. The cultures were washed three times with PBS, incubated in fresh 5% trichloroacetic acid for 20 min at 4°C, washed twice in ice-cold ethanol, and the TCA insoluble material dissolved in 0.1 M sodium hydroxide containing 2% (wt/vol) sodium carbonate overnight at 37°C. Samples of 0.4 ml were neutralized with 1 M HCl and counted in Monofluor.

Autoradiography A modification of the scintillation enhancement method of Durie and Salmon [26] was used. Cell monolayers in 24 well plates were labeled with [³H]thymidine as described above. The washed monolayers were fixed in ethanol:acetic acid (75:25) for 10 min, air dried, and then covered with 0.5 ml of Amplify fluorographic enhancer for 1 min. The monolayers were dried again and overlayed with 0.5 ml K2 emulsion diluted 1:2 with 2% glycerol at 45°C. The excess emulsion was drained off by inversion of the plates, which were then cooled to 10°C for 30 min and air dried. The plates were exposed for 72 h at −70°C in sealed containers.

The emulsion was developed in Kodak D19, fixed, and air dried to prevent distortion during staining with Mayers hematoxylin. Random fields in each autoradiograph were scored at 200 times magnification. A minimum of 2,000 cells were scored per well.

Metabolism of 12(S)-[³H]HETE First passage keratinocytes were cultured in six well plates in medium containing either 10% FCS or 0.5% FCS supplemented with 12-HETE or the co-factor mixture containing cholera toxin, hydrocortisone, and EGF as described for the proliferation assays. At 5 and 12 d the keratinocytes were incubated for 2 h in fresh media of the same composition containing 1 μCi/ml 12(S)-[³H]HETE. Medium was removed from each culture and centrifuged to remove any cellular material. The keratinocytes were scraped into fresh medium with a rubber policeman. Radioactivity in aliquots of the culture supernatants and cell fraction were determined by scintillation counting. The culture supernatants were further analyzed by high performance liquid chromatography (HPLC) on a RP-18 column eluted with a methanol:water:acetic acid gradient at 1 ml/min. Fractions collected from the reverse phase chromatography HPLC were then analyzed on a LiChrosphere Si-60 silica column eluted with a hexane:isopropanol:methanol:acetic acid using a gradient of 98:1.2:0.8:0.1 to 88:7:5:0.1 generated by curve 8 of a Waters HPLC controller. Radioactivity in the HPLC eluent was detected by a Reeve continuous flow monitor.

Materials Racemic 12(RS)-HETE was prepared by photooxidation of arachidonic acid [7]. 12(S)-HETE was prepared by incubating arachidonic acid with human platelets isolated according to the method of Hamberg et al [27]. In each case the 12-HETE was purified by HPLC and purity checked by HPLC on silica and reverse-phase columns and by gas chromatography-mass spectrometry. 12(S)-[³H]HETE was obtained from NEN (DuPont, Stevenage, U.K.).

Tissue culture media and sera were obtained from Gibco-BRL (Paisley, Scotland); tissue culture plastics from Becton-Dickinson (Oxford, U.K.). Human recombinant EGF was a generous gift from

Dr. H. Gregory of ICI Pharmaceuticals, Macclesfield, U.K. Bisbenzamide, calf thymus DNA, ethidium bromide, pronase E, ribonuclease A, and hydrocortisone were obtained from Sigma Chemical Co. (Poole, U.K.); trypsin 3x crystalyzed from Lorne Diagnostics (Reading, U.K.). Monoclonal anti-desmin and anti-vimentin antibodies were from BCL (Lewes, U.K.). Rabbit anti-mouse-HRP was from Dako (High Wycombe, U.K.). Monoclonal antibodies for cytokeratins (AE1, AE2, and AE3) were a generous gift from Dr. T-T. Sun. Autoradiographic emulsion, type K2, was obtained from Ilford (Mobberley, U.K.) and developer D19 from Kodak (Hemel Hempstead, U.K.). Amplify fluorographic enhancing scintillant and methyl-[^{3}H]thymidine (5 Ci/mmol) were supplied by Amersham International (Amersham, U.K.). Monofluor scintillation cocktail was from National Diagnostics (Aylesbury, U.K.).

RESULTS

Keratinocyte Growth in Low Serum Medium Preliminary experiments established that primary or up to third passage keratinocytes plated at 18,000 cells/cm^2 maintained in DMEM containing 10% FCS grew to confluence in approximately 14 d (Fig 1). Reduction of the FCS concentration to 0.5% limited growth, but supplementation of 0.5% FCS with a mixture of cholera toxin, hydrocortisone, and human EGF restored growth to that seen with 10% FCS, demonstrating that the cells retained their ability to proliferate in response to specific exogenous factors. The addition of the mixture of co-factors was used as a positive control for the growth of keratinocytes in 0.5% FCS in subsequent experiments.

Cells cultured in 10% FCS were keratin- and vimentin-positive and desmin-negative. Positive staining for acidic (AE1) and basic (AE3) keratins was similar to the staining pattern of epithelial cells described by Franke et al [28]. Staining was not seen with AE2 anti-keratin antibody. Cells cultured in 0.5% FCS stained similarly to those in 10% FCS. The absence of keratin-negative cells in AE1 and AE2 stained preprations and of desmin staining indicated that fibroblasts, endothelial and smooth muscle cells were not present. Vimentin staining is a characteristic of many cells, including keratinocytes, when cultured in vitro [29].

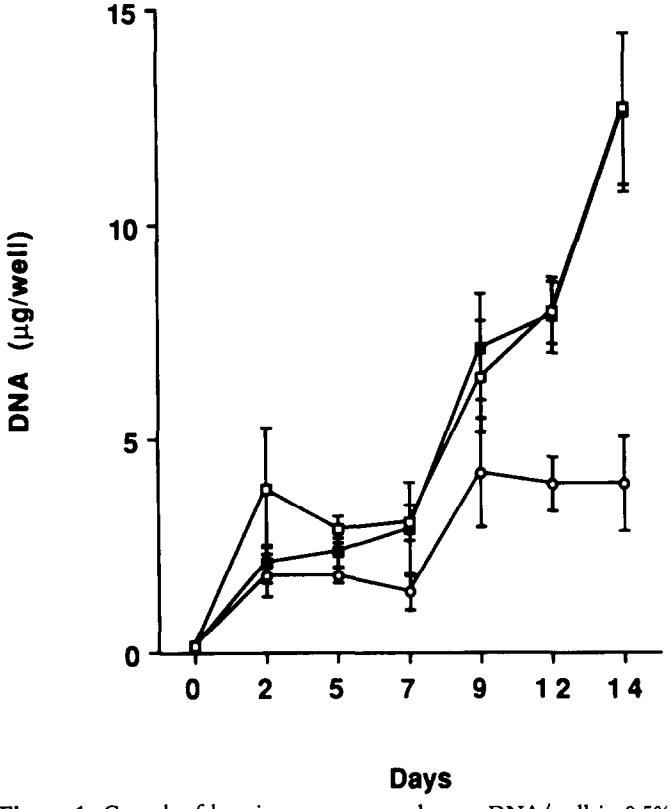

Figure 1. Growth of keratinocytes expressed as μg DNA/well in 0.5% FCS: O—O; 0.5% FCS containing 10^{-10} M cholera toxin, 0.4 μg/ml hydrocortisone, and 10 ng/ml EGF: □—□; or 10% FCS: ■—■, in DMEM. Each point is the mean ± SEM of 4 wells.

Table I. Effect of 12(RS)-HETE on DNA Content and [^{3}H]Thymidine Incorporation in Keratinocytes Cultured for 6 Days

Treatment	DNA (μg/well)	[^{3}H]Thymidine (dpm × 10^{-3}/well)
0.5% FCS	5.03 ± 0.43	96.6 ± 5.0
0.5% FCS + 12(RS)-HETE		
10^{-9}M	5.32 ± 0.51	91.7 ± 1.6
10^{-8}M	4.92 ± 0.44	90.8 ± 3.3
10^{-7}M	5.12 ± 0.55	88.2 ± 4.1
0.5% FCS + CT, HC, and EGF	8.66 ± 0.17[a]	168.7 ± 11.9[a]
10% FCS	7.62 ± 0.67[a]	140.9 ± 5.4[a]

Results are expressed as means ± SEM, n = 4.
CT, HC, and EGF: cholera toxin, hydrocortisone, and EGF co-factor mixture.
[a] $p < 0.05$ compared with 0.5% FCS, Student's *t*-test.

Effect of 12(RS)-HETE on Preconfluent Cultures Second passage keratinocytes were established in 10% FCS for 24 h and then changed to DMEM with 0.5% FCS containing from 10^{-9}–10^{-7} M 12(RS)-HETE, the vehicle for 12-HETE, or the vehicle and the mixed co-factors as a positive control. The cultures received fresh media on days 2 and 4 and were assessed for growth on the sixth day. DNA assay showed that 12(RS)-HETE did not stimulate the proliferation of keratinocytes compared with the control group given the vehicle. The positive control group containing the mixed co-factors showed a significant increase in DNA similar to that obtained with cells maintained in 10% FCS (Table I). The cultures were pulsed with [^{3}H]thymidine for 18 h on day 5 and the uptake measured on the sixth day. Incorporation of [^{3}H]thymidine was not affected by 12(RS)-HETE whether expressed as disintegrations per μg DNA or per well. Keratinocyte cultures stimulated with the mixture of cholera toxin, hydrocortisone, and EGF and cultures exposed to 10% FCS incorporated significantly more [^{3}H]thymidine expressed per well compared with cells in 0.5% FCS, but the specific activities of the labeled DNA (i.e., cpm/μg DNA) were the same.

In a similar experiment, keratinocyte cultures were pulsed with [^{3}H]thymidine for 18 h starting 6 h after the change of media on the fourth day and growth assessed on day 5. Results showing a lack of effect of 12(RS)-HETE on DNA content and [^{3}H]thymidine incorporation were identical to the results for 6-d cultures. [^{3}H]thymidine incorporation, however, expressed per μg DNA as well as per well, was significantly higher in the positive control cultures containing 0.5% FCS supplemented with the mixture of co-factors and the cultures with 10% FCS compared with the other conditions.

There was no evidence for a significant loss of cells from the monolayers under any condition when supernatants were examined by microscopy, or any evidence of change in morphology of keratinocytes exposed to 12(RS)-HETE when examined by phase contrast microscopy.

Effect of 12(RS)-HETE on Growth Over a 12-day Time Course Because there was no evidence that 12(RS)-HETE stimulated proliferation of keratinocytes assessed at single time points in preconfluent cultures, a more comprehensive study was made covering the time course to 12 d when control cultures, containing either 0.5% FCS and the mixture of cholera toxin, hydrocortisone, and EGF or 10% FCS, had reached confluence. Second passage keratinocytes were plated as described above and switched to 0.5% FCS containing 10^{-8}–10^{-6} M 12(RS)-HETE. The media were changed at 2 or 3-d intervals and growth measured at intervals to 12 d. Measurements of DNA confirmed that 12(RS)-HETE had no appreciable effect on cell proliferation at any time point (Fig 2*a*). A small but statistically significant increase in culture DNA was observed for 10^{-7} M 12(RS)-HETE between days 5 and 9, but this was

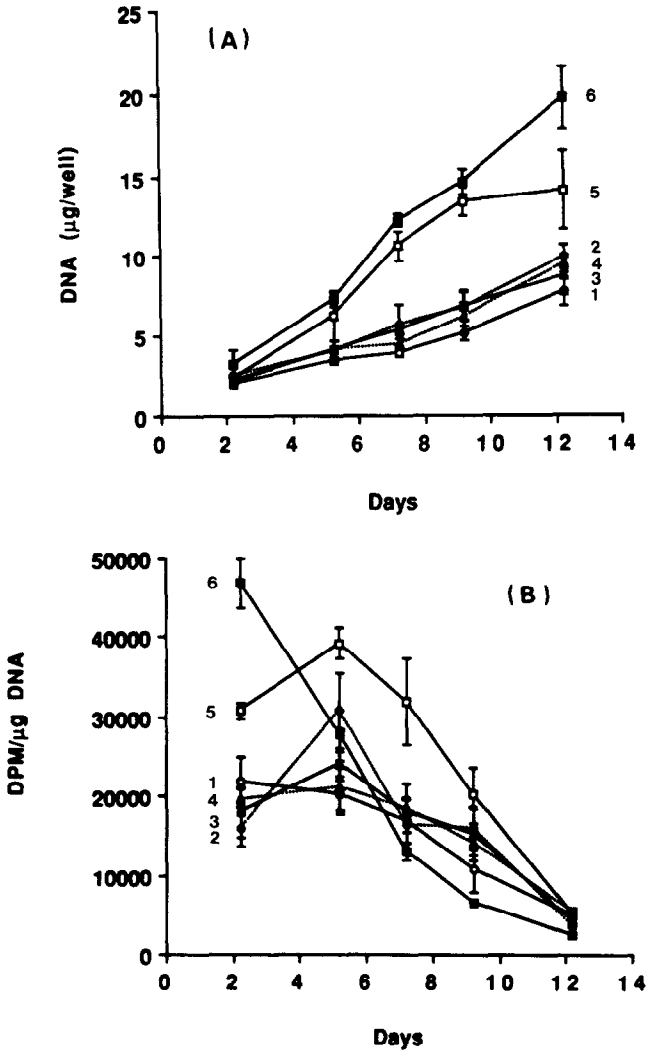

Figure 2. Growth of keratinocytes exposed to 12(RS)-HETE over a 12-day time course. *A*: DNA per well. *B*: [^{3}H]thymidine incorporation per μg DNA. (1) 0.5% FCS: O—O; (2) 0.5% FCS + 10^{-8} M 12(RS)-HETE: ◇ ····· ◇; (3) 0.5% FCS + 10^{-7} M 12(RS)-HETE: Δ—Δ; (4) 0.5% FCS + 10^{-6} M 12(RS)-HETE: ▲ ····· ▲; (5) 0.5% FCS + cholera toxin, hydrocortisone, and EGF: □—□; (6) 10% FCS: ■—■. Each point is the mean ± SEM of 4 wells.

considerably less than that obtained by the control cultures exposed to the mixture of co-factors.

Although the incorporation of [^{3}H]-thymidine in the various culture conditions over this extended time course was more complex, the results still indicated that 12-HETE was without effect on keratinocyte mitogenesis (Fig 2*b*). The only statistically significant increase in thymidine incorporation per μg DNA for cultures with 12(RS)-HETE compared with the controls with no HETE was on day 5 for keratinocytes exposed to 10^{-8} M 12(RS)-HETE. Cultures containing 0.5% FCS and the mixture of co-factors or 10% FCS showed the greatest uptake of thymidine per μg DNA before 5 days; incorporation of thymidine then declined, particularly for cultures containing 10% FCS. At 12 d the incorporation of thymidine was similar for all culture conditions. The same results were obtained in a second identical experiment.

Effect of 12(S)-HETE on Confluent Keratinocytes Grown in Human AB Serum Alternative culture conditions were used to test further the proliferative effects of 12-HETE on keratinocyte growth. Primary keratinocytes were grown to confluence in DMEM containing 10% human AB serum. The cells were then exposed to 10^{-6}–10^{-9} M 12(S)-HETE for 72 h. Control cultures were without 12-HETE or contained the standard co-factor mixture of cholera toxin, hydrocortisone, and EGF.

The DNA content did not change with treatment in any group, and there was there no stimulation of [^{3}H]thymidine incorporation in the cells after exposure to 12(S)-HETE (Table II). Neither the labeling indices nor the number of cells per microscope field changed after incubation with 12(S)-HETE compared with the control cultures.

Incorporation and Metabolism of 12(S)-[^{3}H]HETE Metabolism of 12(S)-[^{3}H]HETE was analyzed under the conditions described above for expanding cultures at 5 d and confluent cultures at 12 d. The results were qualitatively similar but quantitatively the incorporation of 12(S)-[^{3}H]HETE into the cells was about 50% higher for the 12-d cultures. Keratinocytes cultured for 12 d in medium containing 0.5% FCS incorporated 22% of the 12(S)-[^{3}H]HETE during the 2-h incubation. Inclusion of 10^{-7} M 12(RS)-HETE or the mixture of cholera toxin, hydrocortisone, and EGF in the growth media before and during incubation with 12(S)-[^{3}H]HETE had no effect on the incorporation of 12(S)-[^{3}H]HETE, (20% and 25%, respectively). In cultures containing 10% FCS, however, the incorporation of 12(S)-[^{3}H]HETE into the cells was less than 5%.

Analysis of the culture supernatants by reverse phase HPLC showed increased metabolism of 12(S)-[^{3}H]HETE by keratinocytes in cultures containing 0.5% FCS compared with cells in 10% FCS (Fig 3). Before exposure of keratinocyte cultures in 0.5% FCS to either 10^{-7} M 12(RS)-HETE or a mixture of cholera toxin, hydrocortisone, and EGF for 5 or 12 d did not affect the metabolism of 12(S)-[^{3}H]HETE. The peak of radioactivity eluting at 24 min on the reverse phase column co-chromatographed with authentic 12(RS)-HETE and rechromatography on a silica column confirmed its identity as unchanged substrate. Greater than 50% of the 12(S)-[^{3}H]HETE was metabolized by cultures in low serum media compared with less than 15% in the presence of 10% FCS. Metabolism of 12(S)-[^{3}H]HETE did not occur in the absence of cells. Five major and several minor metabolites were detected by reverse phase and silica HPLC, but none of the products co-chromatographed with

Table II. Effect of 12(S)-HETE for 72 Hours on DNA Content, [^{3}H]Thymidine Incorporation, Cell Counts, and Labeling Index of Confluent Cultures of Keratinocytes Grown in Medium Containing 10% Human AB Serum

Treatment	DNA (μg/well)	[^{3}H]Thymidine (dpm/well)	Cells/200x Field	Labeling Index
10% AB serum	18.2 ± 0.38	1030 ± 82	580 ± 30	4.5 ± 1.3
10% AB serum + 12(S)-HETE				
10^{-9}M	17.8 ± 0.97	1148 ± 132	599 ± 36	3.4 ± 1.1
10^{-8}M	19.2 ± 0.46	988 ± 86	658 ± 33	4.3 ± 1.0
10^{-7}M	16.8 ± 0.33	1224 ± 83	622 ± 29	4.4 ± 1.4
10^{-6}M	17.6 ± 0.60	878 ± 56	599 ± 30	5.2 ± 1.1
10% AB serum + CT, HC, and EGF	17.8 ± 0.36	1263 ± 57	594 ± 16	7.8 ± 0.9

Results are expressed as means ± SEM, n = 4.
CT, HC, and EGF: cholera toxin, hydrocortisone, and EGF co-factor mixture.

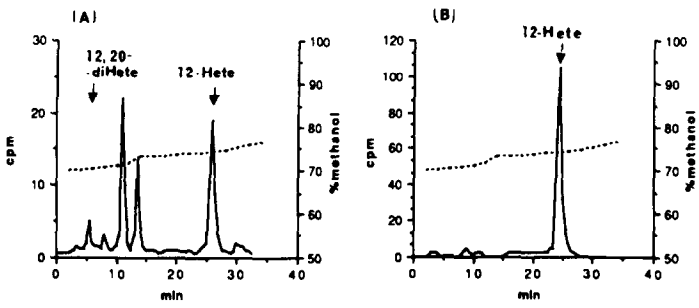

Figure 3. Reverse phase chromatography of 12(S)-[^{3}H]HETE metabolites in supernatants from 12-day cultures of keratinocytes. *A*: 0.5% FCS. *B*: 10% FCS. Elution positions of authentic 12-HETE and 12,20-diHETE are shown. 12(S)-[^{3}]HETE incubated in medium containing 0.5% FCS but no cells gave a trace similar to *B*. Methanol in the solvent gradient: ·····.

authentic 12,20-diHETE, a potential metabolite of 12-HETE by ω-oxidation, in both of the systems.

DISCUSSION

Our evidence suggests that 12-HETE does not increase the proliferation rate of human neonatal foreskin-derived keratinocytes obtained from healthy subjects. Our conclusions that 12-HETE is not a growth factor, based on total DNA measurements in addition to the incorporation of [^{3}H] thymidine, are the same whether expanding or confluent cultures in media containing either fetal calf or human serum were studied. In similar studies we have also been unable to demonstrate that LTB_4, a 5-lipoxygenase product of arachidonic acid, promotes keratinocyte proliferation (manuscript in preparation).

The results of this study fail to substantiate the conclusions of Kragballe and Fallon [13] that 12-HETE stimulates the synthesis of DNA in confluent primary cultures of adult human skin-derived keratinocytes. An explanation for the difference could be that neonatal foreskin-derived keratinocytes are less responsive to 12-HETE than the cells prepared from adult skin. Alternatively, the experimental conditions, which differed in many respects between the two studies, such as medium composition, type and concentration of serum, and nature of the substrate, may affect the response of cultured keratinocytes to 12-HETE. In our experiments we used keratinocytes that had been passaged, making unlikely a contribution from contaminating fibroblasts, melanocytes, and Langerhans cells, which are common in primary isolates of epidermal cells.

Many techniques for keratinocyte culture have been developed to give maximal cell growth [20,30] and as such are unlikely to show further increase in proliferation in response to the inclusion of additional growth factors. In the growth factor assay we developed, keratinocytes were cultured on plastic in medium containing a low concentration of fetal calf serum to give submaximal keratinocyte proliferation. We demonstrated, however, that the low serum medium supplemented with a mixture of specific growth factors could support rapid proliferation of keratinocytes as defined by measurement of increased DNA content of the cultures. In preliminary studies (data not shown) we found that the components of the positive control mixture of co-factors individually promoted keratinocyte proliferation although none was as effective as the complete mixture or 10% FCS. Identification of the cells in low serum medium as keratinocyte was confirmed by immunocytochemical staining. A growth assay similar to ours was used by Wilkinson to demonstrate that serine can stimulate keratinocyte proliferation [31].

In the earlier study by Kragballe and Fallon [13] an increase in the incorporation of [^{3}H]thymidine was presumed to indicate increased synthesis of DNA in cultures exposed to platelet derived 12-HETE. This conclusion, however, was not supported by any change of the DNA content of the cultures. It is necessary to be cautious when interpreting the results of [^{3}H]thymidine incorporation into cells. A cultured breast epithelial cell line showed no change in DNA content on stimulation with estradiol but increased thymidine incorporation by 40% [18]. Potten [32] has reviewed evidence that epithelial cells can store nucleotides without incorporation into DNA. Recently it was shown that human keratinocytes can catabolize thymidine [19]. In general, the measurement of DNA or cell numbers over a time course is the best indice of proliferation of cells in culture.

12-HETE in lesional psoriatic skin was initially identified as the 12(S) isomer [1] but recent stereochemical analysis has shown that a substantial proportion of the 12-HETE in psoriatic scale has the 12(R) configuration [33]. The 12(R) isomer is biologically active, being more potent than 12(S)-HETE in neutrophil chemokinesis [34] and lymphocyte chemotaxis [35] assays. The significance of 12(R)-HETE in psoriasis is not fully understood. As sufficient quantities of the 12(R)-HETE isomer were unavailable we used chemicaly prepared racemic 12(RS)-HETE in several of the growth assays.

Although 12-HETE is reported to cause epidermal hyperplasia when applied to guinea pig ears [36,37], it cannot be concluded that 12-HETE directly stimulated keratinocyte proliferation. Mitogenic factors may be released in the tissues as a result of the inflammatory reaction. At present there is no clear evidence indicating an effect of 12-HETE on epidermal proliferation in human skin in vivo.

We have demonstrated that 12(S)-HETE is metabolized by keratinocytes under the conditions used for the proliferation assays. Metabolism of 12(S)-HETE was less in cultures containing high concentrations of serum, the estimated half-life being 6 h in cultures with 10% FCS and 2 h with 0.5% FCS. None of the metabolites could be identified as 12,20-diHETE, a known metabolite of 12-HETE in polymorphonuclear leukocytes by the ω-oxidation pathway [38]. This data, taken in conjunction with our earlier results that human skin releases only small amounts of 12,20-diHETE in vivo [39], implies that the metabolism of 12-HETE by ω-oxidation is not of significance in human skin. Increased in vitro 12-lipoxygenase activity in uninvolved psoriatic epidermis has been reported [40]. Although the raised levels of 12-HETE in lesional psoriatic skin probably result from increased synthesis due to the higher levels of arachidonic acid substrate [1,2] and lipoxygenase activity, this cannot be confirmed until metabolism has been studied in more detail. Arachidonic acid metabolism by the lipoxygenase pathways is abnormal in psoriasis. Our data from experiments on keratinocytes obtained from neonatal human skin do not support the view that the increase in proliferative activity in psoriasis is likely to result from the mitogenic activity of 12-HETE, although we cannot exclude the possibility that cells from adult skin behave differently. The inflammatory properties of 12-HETE may be of greater significance in this disease.

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